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Inhibition of leucocyte migration by stimulated mononuclear cell supernatants from patients with ischaemic heart disease

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SUMMARY Leucocyte migration inhibition in patients with ischaemic heart disease was evaluated as an assay for progressive myocardial damage. Abnormal results were observed in 50% of patients with ischaemic cardiac disease. The prevalence of abnormal leucocyte migration inhibition was unrelated to clinical presentation, extent of coronary artery disease, or degree of impairment of left ventricular function. Six of the eight patients with unstable angina pectoris and abnormal leucocyte migration inhibition developed life threatening cardiac complications in the follow-up period compared with five patients with unstable angina and normal tests who developed no complications. A similar association between abnormal leucocyte migration inhibition and complications was not observed in patients with angina pectoris or previous myocardial infarction. Thus, leucocyte migration inhibition may be useful as a prognostic marker in unstable angina and may be an important additional variable to identify a high risk subset.

Recent interest has focused on the possible role of immunological factors in the inflammatory response that follows acute myocardial infaction.[1] Current evidence suggests that immune dysfunction may contribute to the pathogenesis of the various types of ischaemic heart disease. Two recent studies have examined the potential role of cell-mediated immunity in patients with ischaemic heart disease, and have indicated that leucocyte migration inhibition to heart extract was present in virtually all patients after myocardial infarction and might persist in some subjects for as long as two years.[2,3] These investigators also evaluated patients with angina pectoris, unstable angina pectoris, and the intermediate coronary syndrome, and presented data that leucocyte migration inhibition might serve as an index of continuing tissue damage in ischaemic heart disease. Our present study extends these observations on abnormalities of cell mediated immunity, particularly in patients with angina pectoris and unstable angina pectoris, and correlates abnormal leucocyte migration inhibition with standard indices of severity of coronary artery disease and prognosis.

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Subjects and methods

Relevant clinical data on the study groups are shown in Table 1. Twelve patients (10 men and 2 women) mean age 53 years, with angina pectoris were included. One patient with angina pectoris had mitral valve prolapse and one the bradycardia-tachycardia syndrome. None of the patients with angina pectoris had had a prior myocardial infarction. All were on nitrates, 10 were on propranolol, and two were taking diuretics for treatment of mild hypertension. Thirteen patients (five men and eight women) mean age 63 years, with unstable angina pectoris were also studied. The diagnosis of unstable angina pectoris was based on the presence of severe, unprovoked, frequently prolonged chest pain, which often did not respond to a single, sublingual glyceryl trinitrate tablet. Two of these patients had a prior history of myocardial infarction. In nine of these patients, angina pectoris preceded the development of unstable angina pectoris. All patients with unstable angina pectoris were admitted to the coronary care unit for exclusion of acute myocardial infarction by serial electrocardiograms and enzyme studies. Eleven patients with unstable angina pectoris were on nitrates and propranolol,

Table 1 *Clinical characteristics of study groups*

Patient group	*No.*	*Mean age (y)*	*Men*	*Women*	*Catheterisation data**
Angina pectoris	12	53	10	2	11/12
Unstable angina pectoris	13	63	5	8	12/13
Myocardial infarction	13	59	8	5	10/13
Cardiomyopathy	13	40	11	2	10/13
Control	12	41	7	5	0/12

*Carried out within six months of LMI assay.

while one had received digoxin for paroxysmal supraventricular tachycardia. Thirteen patients (eight men and five women) mean age 59 years, with a diagnosis of myocardial infarction were also included. Two had myocardial infarction within one week of the study, eight between three and 12 months previously, and in three an interval of greater than one year had elapsed since the infarction. Four had a history of congestive heart failure requiring digitalis and diuretics. Seven were on propranolol, and nine were taking nitrates. Thirteen patients, 11 men and two women, mean age 40 years, with the diagnosis of idiopathic cardiomyopathy were included as a cardiac disease control. All were symptomatic and classified as Functional Class II-III, New York Heart Association. We had previously noted abnormal immunological variables in this disease, though the precise immunological mechanisms were not established.[4 5] These patients were on standard treatment for congestive heart failure, which included digitalis, diuretics, and oral potassium salts. Several of these patients were on warfarin and nitrates. Twelve normal adults (seven men and five women) mean age 41 years, constituted the control group. None of these subjects had a history of cardiac disease.

Cardiac catheterisation and coronary angiography were carried out in 11 of the 12 patients with angina pectoris, 12 of 13 with unstable angina pectoris, 10 of 13 with myocardial infarction, and 10 of 13 with idiopathic cardiomyopathy. Significant coronary artery disease was assumed to be present when narrowing of 75% or greater was present in one or more of the coronary arteries. Impairment of left ventricular function was judged to be present when the left ventricular ejection fraction was less than 50% or when regional or diffuse abnormality of left ventricular wall motion was noted.

LEUCOCYTE MIGRATION INHIBITION

The assay for leucocyte migration inhibition was modified from the methods of Clausen,[6] Gorski *et al.*,[7] and Willoughby *et al.*[8] Blood was collected in preservative-free sodium heparin (10 U/ml) and layered on to a Ficoll-Paque gradient (Pharmacia) in sterile, 50 ml plastic tubes by the method of Boyum.[9] After centrifugation at 400 × *g* for 40 min at 20°C, the mononuclear-rich cell layer at the plasma interface was collected and washed twice with Ca^{++} and Mg^{++} free Hanks's balanced salt solution (HBSS, GIBCO) with centrifugation each time at 250 × *g* for 12 minutes. The cells were diluted to a concentration of 5 × 10^5/ml with RPMI 1640 medium enriched with L-glutamine and 24 mmol HEPES buffer (GIBCO) and supplemented with either 20% AB^+ pooled serum or autologous serum which had been heat inactivated for 30 minutes at 56°C. Cultures were established in microtest plates (Falcon) which contained 0·2 ml/well (10^5 cells). Additional 200 mmol L-glutamine (100 ×) (29·2 mg/ml) (GIBCO) was added to the culture medium, along with 0·1 ml of antibiotic-antimycotic (100 ×) (0·1%) (GIBCO)-penicillin (10 000 units/ml), fungizone (25 μg/ml), and streptomycin (10 000 μg/ml). Each test well contained either 10 μl of Concanavalin A (Con A, Difco), final concentration 20 μg/ml, or normal heart extract containing 6 mg/ml of protein (Lowry method). The plates were incubated at 37°C in a humidified, 5% CO_2 incubator for 72 hours (Con A) or 96 hours (heart extract). Each plate was centrifuged 15 min at 400 × *g*, and the supernatants were collected and stored at −70°C until tested in the second stage. Before storage, control supernatants were reconstituted with Con A or heart extract to serve as unstimulated controls.

Blood from at least three donors was collected in sterile syringes with 10 U/ml of preservative-free heparin for purification of leucocytes. After Ficoll-Paque centrifugation at 400 × *g* for 20 min, the red cell leucocyte layer was collected, diluted with HBSS (2:10) added to 6% dextran (MW 250 000, Sigma) in a ratio of 5:1, and allowed to sediment at a 45 degree angle for 35 minutes at 20°C. The buffy coat was aspirated, washed once, and the remaining erythrocytes lysed quickly with distilled water. The polymorphonuclear leucocytes were then diluted to a concentration of 8 × 10^6/ml with medium 199 with HBSS and L-glutamine. One ml of this mixture was centrifuged at 250 × *g* for 3 min, the supernatant was discarded, and 50 μl of the previously frozen test supernatant was added and incubated for 30 min at 20°C.

Agarose was prepared daily using 1% agarose (Fisher Scientific), 10% heat-inactivated horse serum (GIBCO), 0·17% sodium bicarbonate, and 1·66% antibiotic-antimycotic in medium 199 with HBSS and L-glutamine. Agarose, 4 ml, were placed in a 60 × 15 mm petri dish (Falcon) and allowed to gel. The plates were preincubated in 2% CO_2 immediately before the leucocyte migration inhibition assay. Wells with a 2·5 mm diameter were cut, and 5 μl aliquots of cells and supernatant were placed in each well. Each

assay was performed in triplicate. The plates were incubated for 18 hours at 37°C in 2% CO_2 and were fixed at the end of incubation with 5% glutaraldehyde (Fisher Scientific) and stained with Wright's reagent. Two diameters of each area of migration were measured and averaged. The total area of migration for each triplicate assay and its control was calculated after subtracting the area of the well. The migration index was derived by dividing the mean area of each test migration by the mean area of control supernatant migration, which contained either reconstituted Con A or heart extract. A leucocyte migration index greater than the 90% confidence limit of the normal control group (for Con A stimulated cultures) or less than the 90% confidence limit for heart extract stimulated cultures was considered abnormal.

The specificity of leucocyte migration inhibition factor was evaluated by inhibition of its action in the second stage of the assay with simple sugars. On cells from six normal subjects this factor was inhibited by the addition of 0·1 M N-acetylglucosamine (0·63 ± 0·09, mean and SD of migration index with Con A compared with 0·96 ± 0·04 with Con A plus sugar) and not by the addition of 0·1 M L-fucose (0·57 ± 0·09). Each sugar alone produced no leucocyte migration inhibition in these assays (0·97 ± 0·08 for N-acetylglucosamine and 1·0 ± 0·09 for L-fucose). Student's t test was used to compare the means of response and χ^2 was examined for 2 × 2 tables.

FOLLOW-UP

All patients in this study were followed for three to six months after the initial observation period (Table 2). Information with respect to status was available in 11 of the 12 patients with angina pectoris, all of whom were alive. One patient in this group had undergone coronary artery bypass surgery without complications. Ten of 13 patients with unstable angina pectoris had coronary artery bypass surgery. Three died during the operation or in the immediate postoperative period. Two of the three patients who were not operated on developed congestive heart failure, and one suffered a subendocardial myocardial infarction. Two of the 13 patients with myocardial infarction died with intractable congestive heart failure. One of the four patients in the group who underwent coronary artery bypass surgery developed an intraoperative myocardial infarction. At follow-up, one patient had suffered another infarction while two were being treated for severe congestive heart failure. Of the 13 patients with idiopathic cardiomyopathy, eight were alive at the end of the study period.

Table 2 *Follow-up data in various patient groups*

Patient group	*No.*	*Information available in*	*No. with CABG*	*No. of complications*		
				CHF	*MI*	*Death*
Angina pectoris	12	11	1	0	0	0
Unstable angina pectoris	13	13	10	2	1	3
Myocardial infarction	13	13	4	2	1	2
Cardiomyopathy	13	13	0	0	0	5

CABG, coronary artery bypass graft; CHF, congestive heart failure; MI, myocardial infarction.

Results

Leucocyte migration inhibition to heart extract for all subjects along with the 90% confidence limit of the control group (0·80 migration index) is shown in Fig. 1. All values below the line were considered abnormal (lymphocytes released leucocyte migration inhibition factor on incubation with antigen). Leucocyte migration inhibition was noted in six of 12 patients with angina pectoris (50%), seven of 13 with unstable angina pectoris (54%), six of 13 with myocardial infarction (46%), three of 13 with idiopathic cardiomyopathy (23%), and one of the 12 controls (8%) (Table 3). χ^2 analysis of these data showed no significant difference between the control and idiopathic cardiomyopathy groups, while values calculated for the results between control and angina pectoris, control and unstable angina pectoris, and control and myocardial infarction groups were significant ($p<0·01$). To demonstrate that lymphocytes from the study groups were capable of secreting leucocyte migration inhibition factor in response to a mitogen, comparison incubations were performed with Con A. These results are shown in Fig. 2 together with the 90% confidence limit for the control

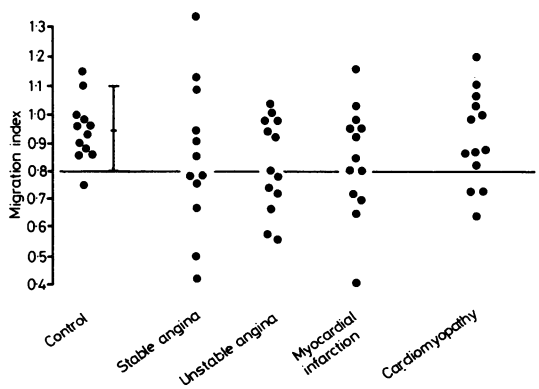

Fig. 1 *Results of assays for leucocyte migration inhibition to heart extract in the study groups. The solid horizontal line at an index of 0·80 was computed as the 90% confidence limit for the control subjects. Values above this line indicate no inhibition of migration (no sensitisation to heart extract) and values below indicate a positive or abnormal result.*

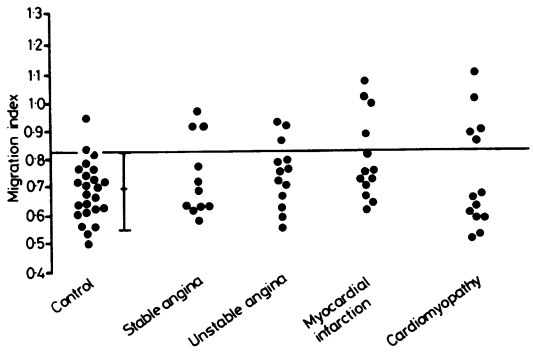

Fig. 2 *Results of assays for leucocyte migration inhibition to Concanavalin A in the study groups. The solid horizontal line at an index of 0·82 was computed as the 90% confidence limit for the control subjects. Values below this line indicate inhibition of migration (response to the mitogen) and values above indicate a negative or abnormal result.*

Table 3 *Results of assay for leucocyte migration inhibition to heart extract for study groups*

Patient group	*Total No.*	*No. abnormal*	*% abnormal*	*p<**
Angina pectoris	12	6	50	0·01*
Unstable angina pectoris	13	7	54	0·01*
Myocardial infarction	13	6	46	0·01*
Cardiomyopathy	13	3	23	0·10
Control	12	1	8	—

*Significant by χ^2 analysis.

group (0·82 migration index). Failure to inhibit leucocyte migration in response to Con A (values above the line) was considered abnormal. Thus, three of 11 patients with angina pectoris (27%), three of 13 with unstable angina pectoris (23%), four of 13 with myocardial infarction (31%), five of 13 with idiopathic cardiomyopathy (38%), and two of 24 control subjects (8%) showed abnormal leucocyte migration inhibition responses to Con A (Table 4). The differences in leucocyte migration inhibition with Con A between control and idiopathic cardiomyopathy, and control and myocardial infarction groups were significant ($p<0·01$), but they were not between control and angina pectoris or unstable angina pectoris groups.

Table 5 shows the leucocyte migration inhibition indices (mean ± SD) to heart extract and Con A in

Table 4 *Results of assay for leucocyte migration inhibition to Concanavalin A for study groups*

Patient group	*No.*	*No. abnormal*	*% abnormal*	*p<*
Angina pectoris	11	3	27	0·06
Unstable angina pectoris	13	3	23	0·08
Myocardial infarction	13	4	31	0·01*
Cardiomyopathy	13	5	38	0·001*
Control	24	2	8	—

*Significant by χ^2 analysis.

this study. The differences in the mean values between the control group and patients with angina pectoris, unstable angina pectoris, myocardial infarction, and idiopathic cardiomyopathy were not significant for either heart extract or Con A.

The results of cardiac catheterisation and coronary angiography in these patients are shown in Table 6. Among the 11 patients with angina pectoris, three had mild or insignificant coronary artery disease; in two of these, coronary artery spasm was excluded by ergonovine maleate administration. Impairment of left ventricular function was judged to be mild in four of 11 patients with angina pectoris and moderate in three. Four of the 11 patients with angina pectoris had no impairment of left ventricular function. All 12 of the patients with unstable angina pectoris showed significant coronary artery narrowing. Impairment of left ventricular function was mild in three of the 12, moderate in eight, and severe in one. All 10 patients with myocardial infarction who underwent coronary arteriography showed evidence of significant coronary artery disease. Impairment of left ventricular function was judged to be mild in two patients, moderate in four, and severe in four. Only one of the 10 patients with cardiomyopathy showed significant coronary artery narrowing, involving one vessel. The haemodynamic studies and left ventricular contrast angiography were consistent with a diffuse cardiomyopathy process in all cases. No significant correlation between an abnormal leucocyte migration inhibition induced by Con A or heart extract, or both, and the severity of the coronary artery disease was noted in patients with angina pectoris, unstable angina pectoris, or myocardial infarction. Similarly, no significant correlation was noted between abnormal leucocyte migration inhibition and degree of

Table 5 *Leucocyte migration indices to heart extract and Concanavalin A in study groups*

	Angina pectoris N = 12	*Unstable angina N = 13*	*Myocardial infarction N = 13*	*Cardiomyopathy N = 13*	*Control N = 12*
Heart extract	0·86 ± 0·26*	0·82 ± 0·16	0·84 ± 0·19	0·92 ± 0·17	0·94 ± 0·11
Concanavalin A	0·73 ± 0·14	0·74 ± 0·12	0·80 ± 0·15	0·72 ± 0·19	0·69 ± 0·10

*Mean ± SD.

Table 6 *Relation between severity of coronary artery disease and results of assay for leucocyte migration inhibition to either Con A or heart extract or both*

Patient group	*No.*	*Severity of coronary artery disease*					*Impairment of ventricular function*				*LMI*	
		*Mild**	*1VD*	*2VD*	*3VD*	*Left main*	*None*	*Mild*	*Moderate*	*Severe*	*Abnormal*	*Normal*
Angina pectoris	11	3	2	3	3	0	4	4	3	0	8	3
Unstable angina pectoris	12	0	0	4	8	0	0	3	8	1	8	4
Myocardial infarction	10	0	1	4	5	0	0	2	4	4	7	3
Cardiomyopathy	10	9	1	0	0	0	0	0	3	7	4	6

VD, vessel disease; Left main, Left main coronary artery; *Patients without significant coronary obstruction (50% narrowing) in any of the vessels.

Table 7 *Correlation between development of complications and leucocyte migration inhibition in patients with unstable angina pectoris*

	Abnormal LMI	*Normal LMI*
Complications*	6	0
No complications	2	5
Total	8	5

*Congestive heart failure, persistent severe angina, death.

impairment of left ventricular function as assessed by cineangiography. Two of the three patients with angina pectoris who had insignificant coronary artery stenosis and normal left ventricular function by cineangiography had abnormal leucocyte migration inhibition. When complications were tabulated and correlated with the immunological variables, abnormal leucocyte migration inhibition was associated with the development of congestive heart failure, myocardial infarction, and death only in the patients with unstable angina pectoris and not in those with idiopathic cardiomyopathy, angina pectoris, or myocardial infarction ($p<0.001$). Six patients with unstable angina pectoris who developed these had abnormal assays for leucocyte migration inhibition either to heart muscle extract or Con A. Two of the seven patients with unstable angina pectoris who did not develop complications also showed leucocyte migration inhibition to either heart extract or Con A (Table 7).

Discussion

It has been previously determined that chronic destruction of tissue may induce abnormal cell-mediated immunity to specific antigens.[10] Wartenberg and Brostoff[2] showed that leucocyte migration inhibition was present to rat heart or rat liver mitochondria in virtually all patients who had experienced a previous myocardial infarction. This in vitro abnormality of cell-mediated immunity was long lasting and could be demonstrated for at least two years after the infarction. The degree or extent of leucocyte migration inhibition in their study bore no relation to the length of time that had elapsed since the myocardial insult. Two of three patients with angina pectoris and one control subject with a prior history of myocarditis also showed leucocyte migration inhibition. Interestingly, these authors studied two patients who showed significant leucocyte migration inhibition to rat heart mitochondria but not to heart extract after laparotomy. They postulated that leucocyte migration inhibition might reflect previous, and perhaps continuing tissue damage or inflammation, and reasoned that this assay might be used in differentiating chest pain that resulted from ischaemic heart disease from that related to non-cardiac origins. Sharma *et al.*,[3] using human heart extract as antigen, also showed significant leucocyte migration inhibition in all patients with myocardial infarction, in 75% of the patients with the intermediate coronary syndrome, and in 25% of those with unstable angina pectoris. No patients with angina pectoris showed leucocyte migration inhibition in this study. These authors noted leucocyte migration inhibition as early as three to four weeks after myocardial infarction and as late as one year after the event. No follow-up data to ascertain the significance of leucocyte migration inhibition in their patients with intermediate coronary syndrome and unstable angina pectoris were available. None of their patients had prior coronary arteriography or necropsy data to confirm the diagnosis and severity of coronary artery disease.

Data from the present study suggest that leucocyte migration inhibition may be observed in approximately 50% of patients with ischaemic heart disease, irrespective of their clinical presentation, extent of the coronary artery disease, or degree of impairment of left ventricular function. Furthermore, patients with other forms of heart disease, such as idiopathic cardiomyopathy, may also develop leucocyte migration inhibition to human heart extract. It is possible that the lesser prevalence with idiopathic cardiomyopathy in the group may reflect the nonhomogeneity of these patients with respect to the aetiology of their impair-

ment. This conjecture is in keeping with our previous observation that abnormal tests for lymphocyte transformation were observed in only 30% of the patients with idiopathic cardiomyopathy.[4] Thus, it appears that leucocyte migration inhibition, as determined by the agarose method, is a relatively sensitive but not a specific assay for the detection of ischaemic myocardial damage. It is probable that any form of cardiac damage, be it ischaemic or inflammatory, may be associated with abnormal leucocyte migration inhibition. The significance of the abnormal leucocyte migration in two of the three patients in the study with angina pectoris associated with minimal coronary artery narrowing and unimpaired ventricular contraction is intriguing. One of these patients had a complete left bundle-branch block and the other had coronary artery calcification in one vessel along with mitral valve prolapse.

It has been suggested that patients with unstable angina pectoris or those with severe angina pectoris and congestive heart failure may have intermittent, slowly progressive myocardial necrosis, which in some cases leads to death.[11,12] At necropsy, no clear histological evidence of acute myocardial infarction may be present, but instead, myocytolysis, coagulation necrosis, and fibrosis are found. Inflammatory cells and granulation tissue, characteristic of acute myocardial infarction, are absent in these cases. During life, the majority of these patients may have uptake of ^{99}M-technetium stannous pyrophosphate consistent with continuing myocardial necrosis that may not be detected by serial electrocardiograms or cardiac enzyme determinations.[13] An excess mortality is found in these patients.[14,15] Though continuing cardiac necrosis was suspected in our patients with unstable angina pectoris, no necropsy data or tissue examinations were available to document this possibility.

The present study suggested that patients with unstable angina pectoris and abnormal leucocyte migration inhibition had a striking tendency to develop severe complications, which included congestive heart failure, myocardial infarction, and death during the follow-up period (Table 7). Thus an abnormal leucocyte migration inhibition may be an important additional variable to identify a high risk subset of patients with unstable angina pectoris. Should future study involving a greater number of patients confirm the low incidence of serious complications in patients with unstable angina pectoris and normal leucocyte migration inhibition, the finding of normal leucocyte migration inhibition and normal pyrophosphate scans could justify a cautious period of conservative treatment rather than immediate early bypass surgery in such patients.

Abnormal leucocyte migration inhibition appears to be a relatively sensitive but non-specific marker of ischaemic myocardial damage. Though there is no direct association between abnormal leucocyte migration inhibition and the extent of coronary artery disease or degree of impairment of left ventricular function, abnormal leucocyte migration inhibition appears to correlate with the development of severe complications in patients with unstable angina pectoris. Further study of this phenomenon may lead to important insights into the immunological mechanisms of ischaemic complications in patients with heart disease as well as provide an important additional prognostic indicator in patients with unstable angina pectoris.

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